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Coupling of the NMDA receptor to neuroprotective and neurodestructive events

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Abstract

NMDA receptors are a subtype of ionotropic glutamate receptor with an important role in the physiology and pathophysiology of central neurons. Inappropriate levels of Ca^{2+} influx through the NMDA receptor can contribute to neuronal loss in acute trauma such as ischemia and traumatic brain injury, as well as certain neurodegenerative diseases such as Huntington's. However, normal physiological patterns of NMDA receptor activity can promote neuroprotection against both apoptotic and excitotoxic insults. As a result, NMDA receptor blockade can promote neuronal death outright or render them vulnerable to secondary trauma. Thus, responses to NMDA receptor activity follow a classical hormetic dose-response curve: both too much and too little can be harmful. There is a growing knowledge of the molecular mechanisms underlying both the neuroprotective and neurodestructive effects of NMDA receptor activity, as well as the factors that determine whether an episode of NMDA receptor activity is harmful or beneficial. It is becoming apparent that oxidative stress plays a role in promoting neuronal death both in response to both hyper- and hypoactivity of the NMDA receptor. Increased understanding in this field is leading to the discovery of new therapeutic targets and strategies for excitotoxic disorders, as well as a growing appreciation of the harmful consequences of NMDA receptor blockade.

Keywords

NMDA receptor; neurodegeneration; neuroprotection; calcium signalling; transcription; oxidative stress

A. Introduction

NMDARs (N-methyl-D-aspartate (NMDA) receptors) are cation-passing channels gated by the neurotransmitter glutamate. They play a central role in synaptic transmission and in mediating synaptic plasticity, learning and memory. NMDARs are also present in oligodendrocytes and astrocytes [1], as well as non neural cells such as osteoclasts [2] and chondrocytes [3] where their function is less well understood. Structurally they are heterotetramers, typically containing two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits. The NR1 subunit is essential for a functional NMDAR, and has several splice variants that can influence the properties of the channel. In addition, NMDAR variety is provided by the four different NR2 subunits (NR2A-D) as well as two NR3 genes (NR3A and NR3B) which can take the place of NR2. The most widely expressed NMDARs contain the obligate subunit NR1 plus either NR2B or NR2A or a mixture of the two. NR2B (and NR2D) is expressed at high levels in early developmental stages which decline post-natally, while NR2A and NR2C expression levels increase [4]. In adults, NR2A is ubiquitously expressed in the brain, NR2B is mostly restricted to the forebrain, NR2C to the cerebellum and NR2D is expressed in small numbers of cells in selected brain regions. However, NR2A and NR2B exist concurrently in the same brain region and participate in the formation of a heterotrimeric NR1/NR2A/NR2B functional NMDARs [5].

The NMDAR channel pore is blocked in a voltage-dependent manner by Mg^{2+} . Synaptic release of glutamate, the main excitatory neurotransmitter in the mammalian CNS (central nervous system), causes Na^+ influx through AMPA receptors in the postsynaptic cell, resulting in partial membrane depolarisation sufficient to lift the Mg^{2+} block. The activated NMDAR is permeable to Na^+ but, crucially, also to Ca^{2+} . This Ca^{2+} mediates most of the physiological effects of NMDAR activity.

At the synapse, the NMDAR is linked to a large multi-protein complex via the cytoplasmic C-termini of NR1 and NR2 subunits [6]. This complex facilitates localisation of the receptor in specific areas, such as the postsynaptic density (PSD), and facilitates connection to a variety of downstream signalling molecules through which many downstream effects of NMDAR activation are mediated [5]. The extreme C-termini of NR2 subunits link to a group of proteins called MAGUKs (Membrane-associated Guanylate Kinases) which include PSD-95, SAP-102 (synapse-associated protein) and PSD-93. These proteins contain several PDZ protein interaction domains through which other proteins are connected. This highly organised structure facilitates signal coupling, by bringing together cytoplasmic signal-transducing enzymes and cell-surface receptors. Moreover, it can bring Ca^{2+} -dependent signalling molecules into close proximity with the site of Ca^{2+} entry at NMDAR. For example, nNOS, in interacting with PSD-95, is brought close to the NMDAR and can thus be efficiently activated by NMDAR-mediated Ca^{2+} influx [7].

NMDARs are essential mediators of many forms of synaptic plasticity and also mediate aspects of development and synaptic transmission [8, 9]. However, when excessively activated, NMDARs can cause cell death in many neuropathological scenarios [10, 11]. During an ischemic episode, extracellular glutamate builds up due to synaptic release and impaired/reversed uptake mechanisms [12, 13] resulting in overactivation of NMDARs [14]. The destructive effects of excessive NMDAR activity are in contrast to the recent findings that survival of several neuronal types is dependent on physiological synaptic NMDAR activity [15, 16]. Responses of neurons to glutamate or NMDA follow a bell-shaped curve: both too much and too little NMDAR activity is potentially harmful [15-17].

B. Clinical trials for stroke with NMDAR antagonists have been unsuccessful

Despite a large body of evidence from animal studies implicating NMDAR activity in neuronal loss following ischemia, the many clinical trials of different NMDAR antagonists for stroke have failed due to poor tolerance and efficacy [15, 18]. The fact that the NMDAR plays a central role in synaptic plasticity and transmission, and learning and cognition, accounts for the undesired psychomimetic and CNS-adverse effects of antagonists [18]. However, trial design may have been erring too far on the side of caution in seeking to avoid psychosis and other CNS-adverse effects, when these side-effects are on-target and not off-target effects. Other issues cloud a clear assessment of NMDAR antagonists, such as numbers of patients within the trials and time taken to administer the drug. With many large pharmaceutical companies having shied away from NMDAR antagonists, these issues may not be resolved any time soon.

Nevertheless, the growing body of evidence that physiological synaptic NMDAR activity exerts a neuroprotective effect has led to suggestions that it may play a role in promoting recovery and preventing delayed neuronal loss in the penumbra [15, 19]. Thus, global NMDAR antagonists may block NMDAR-activated pro-death signals triggered in response to an ischemic challenge, but also interfere with some recovery or preconditioning processes in the penumbra. The anti-excitotoxic effects of NMDAR antagonists have never been in question, but until relatively recently the pro-survival role of the NMDAR was not known

and so antagonists were not tested in contexts that would expose their harmful effects. A classic example of the dichotomous nature of NMDA receptor signalling was reported by the Ikonomidou laboratory who studied the effect of NMDA receptor antagonists as a protective strategy against traumatic brain injury in young rats [20]. They found that while NMDAR antagonists CPP and dizocilpine reduced the primary excitotoxic death proximal to the site of impact, they exacerbated the secondary apoptotic neuronal loss distal to the impact site. Since secondary apoptotic damage was more severe than primary excitotoxic damage, the harmful effects of NMDAR antagonism were significant.

In treating disorders associated with pro-death NMDAR signalling, it may be desirable to block pro-death signalling, without affecting pro-survival signalling or synaptic plasticity. This will require a thorough understanding of the nature of both survival and death pathways triggered by the NMDAR, and the factors that make an episode of NMDAR activity promote survival or death. In this review we will focus on survival and death signalling through the NMDA receptor, exploring newly emerged concepts and the molecules involved.

C. Physiological NMDAR activity can be neuroprotective

In contrast to the adverse effects of excessive NMDAR activity, physiological levels of synaptic NMDAR activity are essential for neuronal survival, since activity blockade was shown to have deleterious effects. Blockade of NMDAR activity *in vivo* caused a decrease in the number of healthy cells, increased density of pyknotic cells and severe deterioration of the dentate gyrus morphology in first week postnatal rat pups [21]. Elimination of NMDA receptor activity *in vivo* causes widespread apoptosis and enhances trauma-induced injury in developing neurons [20-24]. In the adult CNS, NMDAR blockade exacerbates neuronal loss when applied after traumatic brain injury and during ongoing neurodegeneration [25], and prevents the survival of newborn neurons in the adult dentate gyrus [26].

NMDAR signalling to survival has been recapitulated *in vitro* in neuronal cultures [16, 27], allowing scientists to study the underlying signalling events. Network disinhibition using the GABA_A receptor antagonist bicuculline enabled selective studies of synaptic NMDAR activity signalling [28], as opposed to using the unphysiological bath application of glutamate which would activate synaptic and extrasynaptic NMDARs. Bicuculline blockade of the tonic inhibition mediated by GABAergic interneurons promotes action potential bursting which are associated with synaptic NMDAR-dependent Ca^{2+} transients [28]. Pro-survival signalling from the NMDA receptor can involve post-translational modifications of proteins or indeed de novo gene expression. Different signals may have varying importance under different circumstances [29]. We outline below examples of pro-survival pathways triggered by synaptic NMDAR activity, and the pro-survival events that they mediate. Fig. 1 illustrates the pathways described.

C i. Activation of the PI3K-Akt pathway

The PI3K (phosphoinositide-3-kinase) - Akt kinase cascade is an important signalling pathway which contributes to the pro-survival effects of NMDAR activity [29, 30]. It is activated by NMDAR in many neuronal types, but not in all [27]. Briefly, in response to extracellular stimuli, PI3K, which can be activated in a Ca^{2+} -dependent manner by Ca^{2+} /calmodulin [31], catalyses the phosphorylation of the lipid PIP₂ (phosphatidylinositol 4,5-bisphosphate) to PIP₃ (phosphatidylinositol 3,4,5-triphosphate) in the membrane. The kinase PDK1 (phosphoinositide-dependent protein kinase) [32] as well as its substrate Akt/PKB (Protein Kinase B) are recruited to the membrane via their interactions with PIP₃ through their pleckstrin homology (PH) domains. PDK1, along with PDK2 (which is possibly rictor-mTOR- [33]) then phosphorylates and activates Akt. Akt promotes cell survival and growth

via phosphorylation and activation or inactivation of its many targets: Akt phosphorylates and inactivates GSK3 β (Glycogen synthase kinase-3 beta; [34]), an event that also contributes to NMDAR signalling to neuroprotection [35]. Akt mediates further anti-apoptotic events: it inactivates the pro-apoptotic Bcl-2 family member BAD (Bcl2/Bcl-X_L-antagonist causing cell death) by phosphorylation, thus stopping its interaction with the pro-survival Bcl-2 family members Bcl-2 and Bcl-X_L [36]. The JNK/p38 activator ASK1 can also be inhibited by Akt phosphorylation [37] and p53 activity is suppressed by Akt, leading to reduction of Bax expression and of neuronal death [38].

C ii. Suppression of pro-death gene expression

As well as affecting post-translational events that can promote neuroprotection, Akt can also modify gene expression with neuroprotective effect by promoting the phosphorylation and subsequent nuclear export of the FOXO (forkhead box O) subfamily of forkhead transcription factors. FOXOs control the expression of pro-death genes such as Fas ligand [39] and Bim [40], and these are down-regulated by Akt activation. Akt-dependent FOXO export has also been observed in the context of NMDAR signalling [35, 41] which triggers the transcriptional suppression of Thioredoxin-interacting protein (Txnip), a thioredoxin-inhibiting FOXO1 target gene whose product renders neurons vulnerable to oxidative stress [41]. Interestingly, we recently found that FOXO1 itself is a FOXO target gene, meaning that FOXO export triggered by synaptic activity also suppresses FOXO1 gene expression, likely to lead to long-lasting down-regulation of FOXO target gene expression [42].

Importantly, the transcription of the pro-death transcription factor p53 gene has recently been shown to be suppressed by synaptic NMDAR activity [43]. P53 is a transcription factor which regulates a number of pro-apoptotic genes such as Bax and Puma. Knock-down of P53 expression was found to be neuroprotective, preventing mitochondrial depolarisation in response to excitotoxic trauma. In a similar vein, we have observed that key components of the intrinsic apoptosis pathway are transcriptionally repressed by physiologically relevant synaptic NMDAR activity in vitro and in vivo but via a p53-independent mechanism (GEH, unpublished results). Thus, activity-dependent suppression of pro-death genes may represent an important route to neuroprotection, acting in concert with the induction of pro-survival genes, which is discussed below.

C iii. Nuclear Ca²⁺ and activation of CREB (cAMP response element-binding protein)

An important mediator of activity-dependent gene expression is the transcription factor CREB, which binds to the cAMP response element (CRE) [44]. CRE-dependent gene expression is strongly induced by synaptic NMDAR activity [45]. Ca²⁺ influx through the synaptic NMDAR activates the Ras/ERK pathway in the cytoplasm and the nuclear Ca²⁺/calmodulin dependent protein kinases, principally CaMKIV. CaMKIV mediates fast CREB phosphorylation at Ser133, whereas the ERK1/2 pathway promotes CREB phosphorylation in a slower, more long lasting manner [46, 47]. Ser133 phosphorylation of CREB is necessary to recruit CBP (CREB-binding protein), a transcriptional cofactor, to CREB. The transactivation potential of CBP is itself positively regulated by NMDAR activity [48] by a mechanism involving its phosphorylation on Ser301 by CaMKIV [49]. Gene expression mediated by CREB can also be mediated by another family of CREB co-activators, the TORCs (Transducers of Regulated CREB activity, [50, 51] and references therein). TORC2 translocates to the nucleus as a result of the synergistic action of Ca²⁺ and cAMP signalling, through calcineurin-mediated TORC2 dephosphorylation and cAMP-mediated inhibition of the TORC2 kinase SIK2, respectively [50]. In hippocampal neurons TORC1, which is expressed more abundantly than TORC2, senses the coincidence of Ca²⁺ and cAMP signalling and converges this dual signalling to target gene expression by translocating to the nucleus and potentially coactivating CREB [51]. The potential of CREB family-regulated gene

products to promote neuronal survival was first demonstrated in the context of neurotrophin signalling [52, 53] and exogenous overexpression [54]. In addition, studies of mice where CREB and/or CREB family members have been deleted also point to a pro-survival role for CREB *in vivo* both pre- and post-natally [55, 56].

CREB-dependent gene expression is causally linked to the long-lasting phase of activity-dependent neuroprotection against apoptotic insults [29]. This long-lasting phase is dependent on nuclear Ca^{2+} /calmodulin signalling [29], consistent with the known requirement for nuclear Ca^{2+} in CREB activation via CaMKIV [28, 57-59]. Activity-dependent CRE activation in developing CNS neurons also protects against excitotoxic trauma [60] which would otherwise result in rapid cell death resembling necrosis. Expression of the inhibitory CREB family member, ICER, increases in neurons in response to apoptotic and excitotoxic insults and can promote neuronal death [61]. Thus, CREB activation by NMDAR signalling may be important in antagonizing the CREB-inhibiting effects of ICER accumulation.

The importance of NMDAR signalling to CREB in promoting survival *in vivo* is not as well established. Activation of CREB in response to transient ischemic episodes is proposed to contribute to the establishment of ischemic tolerance or preconditioning and may well be mediated by NMDAR activation [62]. Interestingly, resistance to hypoxic/ischemic death in different neuronal populations correlates positively with an ability to sustain active (phosphorylated) CREB [63]: in neurons destined to die CREB is only transiently phosphorylated [63].

The identity of the CRE-regulated gene(s) responsible for long lasting protection against apoptosis is beginning to be understood. Genome-wide profiling studies have identified a large number of CREB target genes, although only a subset of these are induced by CREB-activating stimuli [64-66]. Furthermore, the identity of the genes within the subset is cell-type specific and may be stimulus-specific, so is hard to predict in any particular scenario. A recent study identified two genes that contribute to synaptic NMDAR-dependent neuroprotection [67]: Btg2, a potentially anti-apoptotic CREB target gene, and Bcl6, a transcriptional repressor implicated in suppression of p53, also potentially a CREB target. Both Btg2 and Bcl6 genes confer neuroprotection in the face of apoptotic stimuli like trophic deprivation and staurosporine treatment, and siRNA-mediated knockdown demonstrated that their induction was also necessary for synaptic NMDAR-mediated neuroprotection. While the molecular mechanism of how Btg2 protects is not completely clear, its expression has recently been shown to render neurons resistant to excitotoxicity-induced mitochondrial permeability transitions [43] indicating that it may enhance mitochondrial function, suppress permeability transition, or enhance respiratory capacity. Another CRE-regulated candidate is the pro-survival neurotrophin BDNF (brain-derived neurotrophic factor), which is upregulated by NMDAR activity [45] and is known to promote neuronal survival [27, 68, 69]. NMDAR blockade *in vivo* reduces BDNF mRNA expression and *in vitro* supplementation of neurons with BDNF can rescue them from NMDAR antagonist-induced neuronal death [70]. Still more protective CREB target genes are likely to emerge; it is possible that different genes are responsible for neuroprotection against different types of insult (e.g. apoptotic-like, excitotoxic/necrotic-like, oxidative stress).

C iv. Synaptic NMDAR activity boosts intrinsic antioxidant defences

Correct redox regulation is essential in all cells, especially in post-mitotic cells such as neurons where harmful oxidative damage can accumulate. Oxidative damage and stress occurs when there is an imbalance between production of reactive oxygen species (ROS) and the cell's capacity to neutralize them through its intrinsic antioxidant defenses. Neurons

are particularly susceptible to oxidative damage due to high levels of ROS production (through respiration and metabolism) and relatively low levels of certain antioxidant enzymes, particularly catalase [71, 72]. Oxidative damage is implicated in the pathogenesis of several neurodegenerative diseases as well as acute cerebrovascular disorders [71, 72]. Appropriate redox balance depends on the activity of antioxidant systems. Key among these are the thiol reducing systems based round thioredoxin and glutathione respectively, which are important reducers of many oxidative stressors such as peroxides [72, 73]. The principle source of peroxide is from spontaneous and SOD-catalysed dismutation of superoxide generated in active mitochondria. However, other sources of peroxides exist, including products of metabolic pathways involving oxidases/oxygenases (e.g. monoamine oxidase).

We recently showed that the vulnerability of neurons to oxidative death triggered by exposure to hydrogen peroxide was regulated by synaptic activity acting via NMDA receptor (NMDAR) signalling ([41], see also commentary [41]). Neurons that were experiencing (or had recently experienced) higher levels of synaptic NMDAR activity were far more likely to withstand the oxidative insult than electrically quiet neurons. Neurons experiencing complete NMDAR blockade were highly vulnerable to peroxide-induced apoptosis in vitro, and NMDAR blockade in vivo promoted neuronal apoptosis associated with markers of oxidative damage [41]. It is not completely clear as to why synaptic activity, acting via Ca^{2+} signalling, should act to boost antioxidant defences. One possibility is to protect against increased ROS generation associated with high energy demand. Synaptic activity and activity-dependent plasticity place high energy demands on a neuron which must be largely met through oxidative phosphorylation- a process that generates ROS. Thus, active neurons would ordinarily need to have stronger antioxidant defenses than inactive ones in order to maintain the correct redox balance.

Investigations into the mechanism behind this revealed that synaptic activity exerted a number of changes to the thioredoxin-peroxiredoxin system which contributed to the activity-dependent protection. Synaptic activity enhanced thioredoxin activity and facilitated the reduction of hyperoxidized peroxiredoxins, an important class of antioxidant enzymes. These effects were mediated by a coordinated program of gene expression changes ([41], Fig. 2). Synaptic activity triggered the transcriptional suppression of the thioredoxin inhibitor Txnip, which, as mentioned above, is a FOXO target gene. Furthermore, enhanced reduction of hyperoxidized peroxiredoxins was associated with transcriptional induction of two genes, sulfiredoxin and sestrin 2, whose products are reported to mediate this reaction [74-76]. The induction of sulfiredoxin was mediated primarily through two AP-1 sites, and partly via Nrf2 [77], while sestrin2 was activated via two C/EBP β sites [41]. We did not address which of sestrin 2 or sulfiredoxin was responsible for reducing peroxiredoxin hyperoxidation. However, we recently showed that specific induction of sulfiredoxin is sufficient to prevent peroxiredoxin hyperoxidation in neurons [77], so this is likely to be the most important, particularly as recent doubt has been cast on the ability of sestrin 2 to catalyze this reaction [78]. In any case, induction of one (or both) of these genes cooperated with the suppression of Txnip in boosting antioxidant defenses. The gene expression changes described above did not account for all the antioxidant effects of synaptic NMDAR activity. Work is ongoing in the laboratory to investigate another major antioxidant system which is subject to activity-dependent enhancement-that centred on glutathione (GH, unpublished observations).

D. NMDAR-mediated cell death

A high and prolonged rise in the extracellular glutamate concentration kills central neurons [79]. The NMDAR is an important mediator of glutamate-induced excitotoxicity [80] due in part to its high permeability to Ca^{2+} and incomplete desensitisation. Pathological activation

of NMDARs is a major cause of neuronal death following acute excitotoxic trauma such as brain ischemia, hypoxia and mechanical trauma [10]. Chronic neurodegenerative diseases may also be associated with excessive NMDAR activation [81, 82]. Strong Ca^{2+} influx through the NMDAR triggers a number of intracellular events that lead to neuronal death, several of which are outlined below and in Fig. 3.

D i. Mitochondrial dysfunction and reactive oxygen/nitrogen species generation

Mitochondrial dysfunction caused by excessive Ca^{2+} uptake by the mitochondria through the potential-driven uniporter is a key event in severe excitotoxicity [83, 84]. The mitochondrial membrane gets depolarised due to this uptake, which inhibits ATP production, and can even cause depletion of cytosolic ATP due to reversal of the mitochondrial ATPase. This loss of ATP further limits the ability of the neuron to regulate intracellular Ca^{2+} levels. It had been generally accepted that mitochondrial Ca^{2+} uptake can also promote ROS production, triggering further mitochondrial damage and delayed Ca^{2+} deregulation (DCD). However, recent studies have made the case that enhanced ROS production following a strong excitotoxic insult occurs after DCD, and that most superoxide production is in any case non-mitochondrial in origin [84, 85]. This does not rule out a role for oxidative stress, since prior oxidative stress greatly potentiates NMDAR-mediated toxicity, possibly by reducing mitochondrial ATP supply [86]. Recent evidence for a role for ROS generation upstream of NMDAR-mediated cell death also focused on a non-mitochondrial source: NADPH oxidase (NOX). It was reported that activation of NADPH oxidase is a major source of superoxide production by NMDA receptor overactivation, and that inhibiting this process was neuroprotective [87].

NMDAR activity-regulated overactivation of the Ca^{2+} -dependent nNOS (neuronal nitric oxide synthase) also has toxic downstream responses, including mitochondrial dysfunction, p38 mitogen-activated protein kinase signalling and TRPM (transient receptor potential melastatin) channel activation [88]. nNOS is bound by its N-terminal PDZ domain to the post-synaptic density protein PSD-95, which brings it in the proximity of the synaptic NMDAR that is also bound to PSD-95 via the extreme C-terminal amino acids of its NR2 subunit (its so-called PDZ ligand). nNOS activation leads to Nitric Oxide (NO) production, which in excess can be toxic both on its own and when combined with other reactive oxygen species (ROS) such as superoxide to form ONOO^- (peroxynitrite). Both NO and ONOO^- can damage cellular components, inhibit mitochondrial respiratory chain enzymes and promote mitochondrial depolarisation [10]. ONOO^- also damages DNA, resulting in single strand breaks and overactivation of the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP-1). Excessive PARP-1 activation can promote neuronal death by depleting cellular NAD^+ levels and also by triggering the mitochondrial release of apoptosis activating factor, (AIF) [89, 90]. Another important target of NMDAR signalling to NO production in excitotoxicity is the cation channel TRPM7 [91]. NMDAR-dependent Ca^{2+} influx triggers both NO production via nNOS activation and superoxide production via mechanisms described above, which combine to form ONOO^- , an activator of TRPM7. Since TRPM7 itself passes Ca^{2+} , this results in a positive feedback loop.

D ii. Calpain activation

As well as promoting mitochondrial dysfunction and ROS generation, excessive Ca^{2+} influx also causes efflux to be impaired [92, 93]. In neurons, Ca^{2+} exit from the cell is achieved through the plasma membrane Ca^{2+} ATPase pump (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCXs). Calpains, Ca^{2+} -dependent proteases, are activated by the excessive NMDAR-mediated Ca^{2+} influx and cleave a major isoform of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX3) impairing its function in cerebellar granule neurons (CGNs) [92]. The PMCA, which would utilise the energy from ATP hydrolysis to transport Ca^{2+} across the

plasma membrane, is inactivated by excitotoxic insults via mechanisms attributed to both caspases [94] and calpains [93]. Other important calpain targets have recently been revealed: the metabotropic glutamate receptor mGluR1 α [95] and striatal-enriched protein tyrosine phosphatase (STEP) [96]. NMDAR-dependent, calpain-mediated truncation of the mGluR1 α C-terminal tail does not disrupt coupling to Gq protein activation, but results in other altered signalling properties of the receptor, including a disruption of PI3K-Akt signalling, as well as relocating the receptor from dendrites to axons [95]. The functional result of this truncation is that while wild-type mGluR1 α is neuroprotective against NMDAR excitotoxicity, the truncated form has the opposite effect. NMDAR-mediated calpain cleavage of STEP led to the sustained activation of the stress-activated pro-death kinase, p38 (see below). Furthermore, inhibition of STEP cleavage is neuroprotective and suppresses p38 activation [96].

D iii. Stress-activated protein kinases

Stress-activated protein kinases (SAPKs) are another class of signalling molecules that are implicated in NMDAR-dependent cell death [97-101]. In CGNs, NMDAR-dependent excitotoxic death relies upon p38 activation, leading to caspase-independent cell death [97, 99]. In cortical neurons another SAPK family, the c-Jun N-terminal kinases (JNKs) contribute to NMDAR-dependent cell death in vitro and in vivo as well as p38 [100, 101]. New components of the excitotoxic cell death pathway signalling to p38 are emerging; Rho, a member of the Rho-family of GTPases, was recently found to contribute to glutamate-induced p38 α -dependent excitotoxic neuronal death [102]. As mentioned above, calpain cleavage of STEP has also been implicated in p38 activation [96]. In addition, studies in CGNs and cortical neurons have reported that NMDAR-dependent p38 activation is dependent on activation of nNOS, which is associated with the NMDA receptor signaling complex through its association with PSD-95. PSD-95 is itself linked to NMDARs through interaction with the PDZ ligand of the NR2 subunit. Disruption of NR2 PDZ ligand:PSD-95 interactions that can uncouple the NMDAR from NO production also inhibit p38 activation [101, 103]. Interestingly, disruption of interactions between the NR2 PDZ ligand and downstream proteins such as PSD-95 has a remarkably specific effect on the p38 pathway. NMDAR signalling to pro-survival pathways resulting in activation of Akt or of CREB are not interfered with [101, 104]. However, the neuroprotection afforded by such a strategy is imperfect: activation of JNK does not require interactions with PSD-95. Indeed, NMDAR-induced death via JNK can be reconstituted in non-neuronal cells simply by expressing functional NMDARs [101]. The mitochondrially targeted antioxidant MitoQ [105] inhibited NMDAR-activated JNK signalling in cortical neurons, potentially placing ROS generation upstream of JNK activation [101].

D iv. So many pathways to excitotoxic cell death?

The myriad potential routes to NMDAR-mediated excitotoxicity begs the question as to which is the most important or dominant mechanism. While cell-type specific differences may offer a partial explanation, another important consideration is the intensity of the excitotoxic insult [106]. Extremely high and sustained levels of NMDAR-induced Ca²⁺ influx will likely cause rapid mitochondrial dysfunction and bioenergetic failure leading to DCD. On the other hand, incomplete mitochondrial depolarisation or depolarisation of only a subpopulation of mitochondria may lead to the release of apoptotic factors such as cytochrome c or AIF, and the activation of apoptotic-like biochemical cascades. Activation of SAPK-dependent neuronal death may also be associated with more modest excitotoxic insults near the threshold of toxicity, and take neurons down a more delayed route to death. Consistent with this, peptide inhibitors of JNK and also of NR2 PDZ ligand interactions are neuroprotective in excitotoxic models in vitro and in vivo when administered after the insult [7, 100]. Furthermore, the protective effects of these peptides can be overcome by increasing

the severity of the toxic insult [101], as can neuroprotection by pharmacological inhibition of p38 [98]. Another consideration is that studies on apparently different mechanisms of excitotoxicity may in some cases involve different parts of the same pathway, as illustrated in Fig. 3. For example, NMDAR-dependent generation of oxidative/nitrosidative stress can lead on to mitochondrial dysfunction, as well as SAPK and PARP-1 activation. Similarly, calpain activation acts as a signalling hub for many pro-death events, including p38 activation, disruption of Akt activation, and Ca^{2+} dyshomeostasis.

E. Oxidative stress plays an important role at both ends of the toxicity curve

As described in the sections above, blockade of NMDAR activity in vivo promotes neuronal death associated with oxidative damage [41]. In vitro, NMDAR blockade also renders neurons vulnerable to oxidative insults through the weakening of antioxidant defenses [41]. At the other end of the toxicity curve, excessive NMDAR activity promotes free radical production and oxidative stress in vitro and in vivo [87, 88]. Moreover, exogenous oxidative stress renders neurons particularly vulnerable to NMDAR-induced death [86]. The involvement of oxidative stress in neuronal loss due to both hyper- and hypo NMDAR activity raises the possibility that a single intervention aimed at boosting intrinsic antioxidant defenses could inhibit both forms of neuronal death. This might have use in scenarios where NMDAR activity appears to be having both pro-death and pro-survival effects, such as the traumatic brain injury model described above [20]. In this instance, enhancing antioxidant defenses may not only reduce excitotoxic neuronal death, but could also help mitigate against any harmful effects from co-administered NMDAR antagonists.

F. What determines if an episode of NMDAR activity is neuroprotective or excitotoxic?

As outlined above, there is a multitude of ways in which NMDAR-dependent Ca^{2+} influx can convey the signal downstream to either confer neuroprotection or to trigger programmes of cell death. A key issue, potentially relevant for designing clinically tolerated drugs against neurodegenerative conditions caused by excitotoxicity, is how the distinction between what pathway will be followed is made and what factors affect this. A number of possibilities are presented here.

F i. Stimulus intensity

The magnitude of activation, be it intensity or duration, is very important in determining the nature of the response to an episode of NMDAR activity. The classical bell-shaped curve model of the neuronal response to NMDA or glutamate contends that intermediate, modest/physiological NMDAR activity levels can promote neuroprotection whereas too low or too high pathological NMDAR activity promotes cell death. This implies that the effectors of the pro-survival Ca^{2+} signalling have a higher affinity (or considerably lower requirements for Ca^{2+}) than the Ca^{2+} effectors of death. Therefore the Ca^{2+} concentration threshold for activating pro-survival signalling by PI3K, ERK1/2 and CaMKIV must be lower than that necessary to trigger toxic levels of calpain activation, mitochondrial Ca^{2+} uptake or NO production. The key Ca^{2+} -dependent components of the survival pathways are mainly activated not by Ca^{2+} directly, but by Ca^{2+} /Calmodulin. Calmodulin is a ubiquitous Ca^{2+} binding protein which changes conformation when it binds to Ca^{2+} . Ca^{2+} /Calmodulin then activates a number of downstream signals, including CaMKIV, PI3K and upstream activators of ERK1/2. As a physiological sensor of elevated Ca^{2+} levels, calmodulin is designed to be activated by relatively modest increases in Ca^{2+} . In contrast, central mediators of NMDAR-dependent cell death, calpains, are not Ca^{2+} /Calmodulin dependent

but are activated by Ca^{2+} directly, and require higher levels to be fully induced [107]. The same can be said of the mitochondrial uniporter, which senses Ca^{2+} directly and is fully activated only by high concentrations [108]. Consistent with this, when increasing doses of NMDA are applied to neurons, only the higher, toxic doses evoke sustained loss of mitochondrial membrane potential and increases in mitochondrial Ca^{2+} [35]. Another Ca^{2+} -dependent promoter of neuronal death, nNOS, is Ca^{2+} /Calmodulin-dependent and indeed plays important roles in non-pathological signalling processes such as synaptic plasticity. However, nNOS is also regulated by phosphorylation, and a recent study showed that, in contrast to non toxic stimuli, high levels of glutamate fail to trigger an inhibitory phosphorylation event that ensures that nNOS activation is transient [109]. Thus, nNOS becomes excessively active and contributes to excitotoxic cell death [109].

F ii. NMDAR location

Aside from stimulus intensity, the location of the NMDAR may also profoundly affect the signals that emanate from the NMDAR. Developing neurons have sizeable pools of NMDARs at extrasynaptic, as well as synaptic locations, which signal very differently. Ca^{2+} influx dependent on intense synaptic NMDAR activation is well tolerated by cells whereas equivalent activation of extrasynaptic NMDARs, either on their own or in the presence of synaptic NMDAR activation, is less well tolerated, triggering mitochondrial membrane potential and cell death [45]. The findings from this study were based on hippocampal neurons and have since been recapitulated independently in cortical neurons [110].

Synaptic vs. extrasynaptic NMDAR regulation of survival and death signals—

Differential synaptic vs. extrasynaptic NMDAR effects extend to several signal pathways. While synaptic NMDAR activity strongly induces CREB-dependent gene expression, extrasynaptic NMDARs are coupled to a dominant CREB shut-off pathway that causes CREB dephosphorylation of its activator site (serine 133)[45]. The developmentally regulated mechanism of this is discussed elsewhere [111, 112]. In addition to signalling to CREB, it has also been shown that there is opposing regulation of the ERK1/2 pathway by synaptic and extrasynaptic NMDARs in hippocampal neurons: synaptic NMDARs activate the ERK pathway whereas extrasynaptic NMDARs evoke ERK inactivation [113]. Furthermore, only synaptic NMDAR activity is able to promote sustained activation of the PI3K-Akt pathway [41].

This differential signalling was at apparent odds with the long-standing observation that low levels of bath-applied NMDA or glutamate are neuroprotective: these stimuli will activate both synaptic *and* extrasynaptic NMDA receptors, and yet the pro-death extrasynaptic NMDA receptor-dependent signal is clearly not dominating. The anti-apoptotic effects of low doses of NMDA or glutamate have been demonstrated in several neuronal cell types in vitro, including cerebellar granule cells and hippocampal neurons [114, 115]. Protection against excitotoxic trauma can also be induced by chronic exposure of neurons to a sub-toxic dose of glutamate or NMDA [116-118] via a mechanism thought to involve CREB activation and BDNF synthesis [119, 120]. In addressing this apparent paradox, we found in neurons exposed to low protecting doses of NMDA, synaptic NMDAR signalling is able to dominate because synaptic NMDARs are preferentially activated due to the NMDA causing a dramatic increase in action potential firing [35]. This enhanced firing mediates the NMDA-induced pro-survival signalling to Akt, ERK1/2 and CREB. In contrast, higher, toxic doses of NMDA strongly suppress firing rates and hence do not favour synaptic NMDAR activation [35].

Recently a molecular mechanism was proposed to explain the specific shut-off of CREB by extrasynaptic NMDAR activity, centred on Jacob, a binding partner of the neuronal Ca^{2+} binding protein caldendrin [121]. Jacob, when localized in the nucleus, causes CREB

inactivation and also promotes the loss of synaptic contacts. Jacob contains a nuclear localisation signal and its nuclear import requires importin α binding. This binding and nuclear import is competed by caldendrin in a Ca^{2+} -dependent manner. Interestingly it was reported that synaptic, but not extrasynaptic NMDAR activity promoted caldendrin-dependent retention of Jacob outside of the nucleus. Extrasynaptic NMDAR activity promoted the efficient nuclear accumulation of Jacob, with subsequent deleterious effects [121].

A study involving genome-wide expression analysis has also extended our understanding of synaptic vs. extrasynaptic signalling [67]. While synaptic NMDARs activated a number of pro-survival genes (including the aforementioned Btg2 and Bcl6), extrasynaptic NMDARs failed to do this. Moreover, bath activation of all (synaptic and extrasynaptic) NMDARs failed to suppress Txnip expression or induce expression of sulfiredoxin or sestrin 2, and failed to protect neurons from oxidative stress [41]. Not only do extrasynaptic NMDARs fail to activate pro-survival changes in gene expression, they also couple to expression of a gene Clca1 which kills neurons [67].

Another very recent study demonstrated that activation of extrasynaptic, but not synaptic NMDAR activity promoted calpain activation, an important contributor to excitotoxic cell death. Calpain activation resulted in the cleavage of not only NCX3 (a previously reported substrate [92]) but also the tyrosine phosphatase STEP [96]. STEP cleavage leads to it being unable to interact with its normal substrates, including p38, and so leads to sustained activation of pro-death p38 signalling. A cell-permeable peptide containing the STEP cleavage site of calpain both blocked cleavage of endogenous STEP and was neuroprotective in the face of extrasynaptic NMDAR activity [96].

Differential synaptic vs. extrasynaptic NMDAR signalling: when and how?—

Chronic exposure of neurons to glutamate and activation of extrasynaptic NMDARs is unlikely to occur under normal physiological conditions. However, it may occur under pathological conditions such as brain injury, or during hypoxic/ischemic (H/I) insults where neuronal glutamate transporters operate in reverse [12] thereby pumping glutamate out of the cells into the extracellular medium, building up in the restricted extracellular space. Reversed uptake by glial cells can also trigger neuronal death through activation of extrasynaptic NMDARs [122]. Neurons may also be exposed to elevated ambient levels of glutamate through release from nearby dying cells. Consistent with all this, H/I conditions cause a NMDAR-dependent CREB inactivation [45] and induction of the Clca1 gene [123]. Given the role of CREB in controlling several pro-survival genes, this shut-off may contribute to the slowly progressing apoptotic death of neurons (in response to excitotoxic trauma) that are exposed to toxic levels of glutamate that are insufficient to cause immediate necrotic cell death [124-126].

The molecular basis for the apparent differences in synaptic/extrasynaptic NMDAR signalling could be due to differences in the composition of the NMDAR signalling complexes as opposed to the location of the receptors per se. For example, since caldendrin is localised to the post-synaptic density, it has been postulated that only trans-synaptic activation of synaptic NMDARs trigger high enough local Ca^{2+} transients to promote caldendrin-mediated retention of Jacob [121]. Differential localisation of a death effector has also been reported: p38 was found to be enriched at extrasynaptic membranes compared to synaptic fractions, while other MAP kinases, ERK1/2, were not [96]. Another contributing factor could be the way in which these distinct pools are activated: brief saturating activation in the case of trans-synaptic activation of synaptic NMDARs vs. chronic low level activation of extrasynaptic NMDARs by bath/ambient glutamate.

Differences in the properties of intracellular Ca^{2+} transients evoked by these different stimuli could differentially affect signalling, even if the overall Ca^{2+} load were similar.

F iii. Does subunit composition matter?

While extrasynaptic NMDARs are preferentially enriched with NR2B-containing NMDARs, there is little evidence that differences in subunit composition are dramatic enough to explain the effects observed. However, a recent study has contended that NR2B-containing NMDARs tend to promote neuronal death, irrespective of location (synaptic or extrasynaptic) while NR2A-containing NMDARs promote survival [127]. The cytoplasmic c-terminal tails of NR2A and NR2B are large, show considerable sequence divergence and have been reported to differentially associate with signalling molecules [128], offering scope for subunit-specific routes to survival/death. The investigation of subunit-specific differences in NMDAR signalling is hampered by the lack of a NR2A-specific antagonist that is sufficiently selective to discriminate in the physiological scenario of trans-synaptic stimulation [129, 130]. The antagonist NVP-AAM077 has some selectivity for NR2A-containing NMDARs but studies disagree as to the degree of selectivity, possibly because of some laboratories studying rodent NMDARs and others human NMDARs expressed in oocytes [130, 131]. However, it is likely that the difference in the equilibrium constants (NR2A vs. NR2B) is not sufficient to discriminate between NR2A-containing or NR2B-containing NMDA receptors being trans-synaptically activated [130]. Moreover, NR2A-NMDARs have been shown to be capable of mediating excitotoxicity [132], and NR2B-NMDARs have been shown to be capable of mediating both pro-survival and pro-death signalling, depending on the stimulation paradigm [133]. As such, the issue of whether the NR2 subunit influences whether an NMDAR signals to survival or death (controlling for the amount of Ca^{2+} passed) remains unresolved.

G. Concluding remarks and future perspectives

The pro-survival role of synaptic NMDARs under physiological conditions and especially during CNS development, points to the potential dangers of NMDAR antagonism during this period in humans [134]. Several paediatric/obstetric anaesthetics and anticonvulsants reduce NMDAR activity directly or indirectly; in addition, *in utero* exposure to NMDAR-antagonistic recreational drugs such as phencyclidine, ketamine and ethanol pose significant risks [134]. A greater knowledge of the mechanism of NMDAR-dependent neuroprotection not only increases our understanding of this important pro-survival pathway, but also points to ways in which it might be mimicked for therapeutic effect (for example to mitigate against the harmful effects of NMDAR antagonists).

In the mature CNS, and despite evidence from animal studies implicating the NMDAR in ischemic brain damage, clinical trials of NMDAR antagonists for stroke have failed due to poor tolerance and efficacy [15, 18]. The important role of NMDARs in the CNS may mean that for any antagonist, the maximal tolerated dose is lower than the therapeutically effective dose, since many unacceptable side-effects are on-target effects. Evidence that NMDAR activity can exert a neuroprotective effect has led to suggestions that it may even promote recovery post-reperfusion, and prevent delayed neuronal loss in the penumbra [15, 19]. As such, antagonists may be protective early in the insult but not later on, which indicates a potential need for more specific anti-excitotoxic strategies where the effects on pro-survival NMDAR signalling are considered. One promising strategy is the use of antagonists which preferentially block excitotoxic NMDARs in excitotoxic scenarios, or which are activated only in the pathological state (so-called pathologically activated therapeutics [135]). As an example of the former, memantine is a clinically well-tolerated NMDAR antagonist with pharmacological properties suited to the blockade of chronic NMDAR activity in pathological scenarios without interfering with normal synaptic function [135].

As described in this review, another approach involves targetting a site of action downstream of NMDAR activation, several of which have been described above. For example, peptide-mediated inhibition of JNK, of STEP cleavage, of mGluR α cleavage and of NR2 PDZ interactions have all been shown to have neuroprotective effects in the face of excitotoxic insults [7, 95, 96, 100]. The precise pathway, or pathways that mediate NMDAR-dependent cell death in ischemia may depend on the neuronal subtype, the severity and duration of the episode, and the position of the neuron within the lesion (infarct core or penumbra). Thus, it may be that targeting JNK signalling or NO production may ameliorate neuronal loss in some scenarios, but that blocking TRPM7 activation, or preventing the cleavage of specific calpain targets is more effective in others. As such, the selective targeting of multiple pro-death pathways downstream of the NMDARs may represent an effective and well-tolerated therapeutic strategy.

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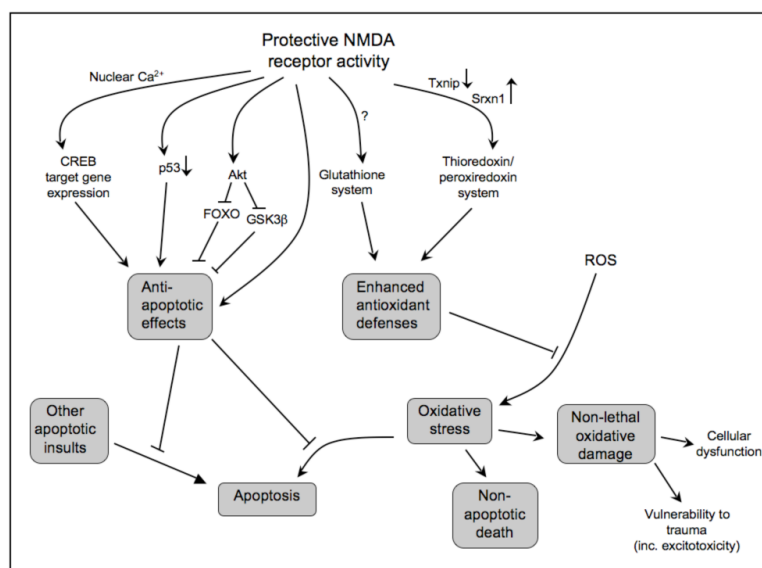


Fig. 1. Neuroprotective signalling by synaptic NMDAR activity

Some examples of anti-apoptotic and antioxidant signalling events that can promote neuroprotection. See text for details.

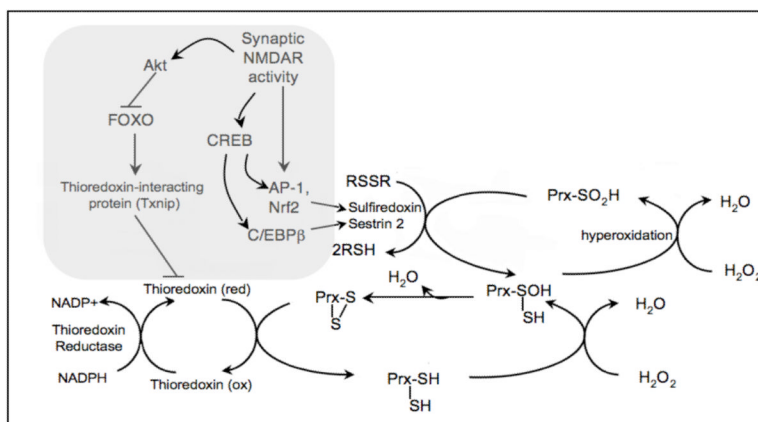


Fig. 2. Synaptic NMDAR activity boosts intrinsic antioxidant defences

A schematic showing impact of activity-dependent changes in gene expression on the thioredoxin-peroxiredoxin cycle (events shaded in grey). Briefly, detoxification of peroxides is mediated through the transfer of reducing equivalents from NADPH to peroxides via the redox-active sulfhydryl (-SH) groups of thioredoxin and peroxiredoxins (Prx, the two-cysteine peroxiredoxin major subtype is shown here). The redox active -SH group of Prx is oxidized to -SOH by peroxides. Ordinarily this converts to a disulfide bond upon reaction with the resolving cysteine -SH group, which is in turn reduced by thioredoxin, returning Prx back to its reduced form. If levels of peroxide get too high or thioredoxin/peroxiredoxin activity too low, the -SOH group becomes hyperoxidized to -SO₂H (cysteine sulfinic acid) which is not a substrate for the resolving cysteine or for thioredoxin. Instead, sulfiredoxin (and possibly sestrin 2) catalyses the reduction of hyperoxidized Prx-SO₂H and returns it to the thioredoxin cycle. Synaptic activity triggers the events shown (and described in the text) to both enhance thioredoxin activity and boost the reduction of hyperoxidized Prxs.

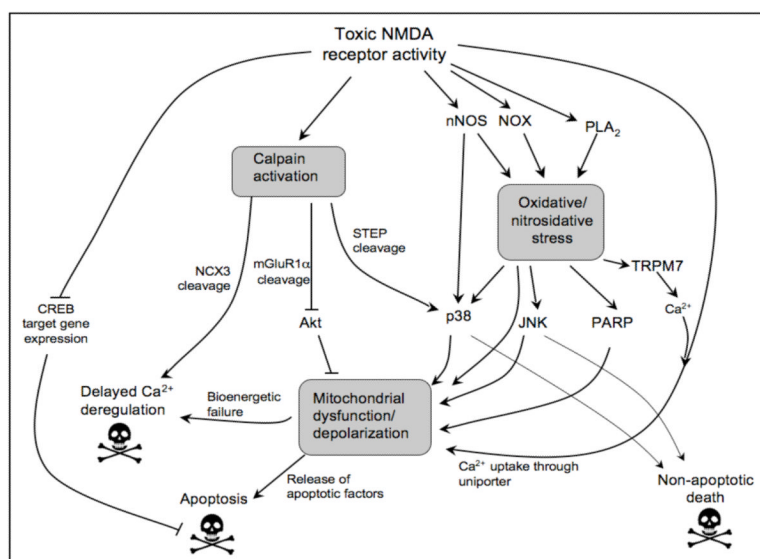


Fig. 3. Neurodestructive signalling by NMDAR activity

Some examples of excitotoxic signalling events promoted by NMDAR activity. Note that some of these events, such as CREB inactivation, calpain-mediated STEP cleavage and disruption of mitochondrial membrane potential are preferentially triggered by chronic extrasynaptic NMDAR activation, rather than trans-synaptic activation of synaptic NMDARs. See text for details.